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Award Number: DAMD17-03-1-0383

TITLE: The Effects of Deregulated Cyclin E Expression in
Mitosis: A Role in Breast Tumorigenesis

PRINCIPAL INVESTIGATOR: Jamie M. Keck

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (1 May 2003 - 30 Apr 2004)	
4. TITLE AND SUBTITLE The Effects of Deregulated Cyclin E Expression in Mitosis: A Role in Breast Tumorigenesis			5. FUNDING NUMBERS DAMD17-03-1-0383	
6. AUTHOR(S) Jamie M. Keck				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Burnham Institute La Jolla, California 92037 E-Mail: jkeck@sripps.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The objective of this study is to identify the mechanism through which deregulated expression of cyclin E leads to chromosome instability, first observed by Spruck et al. in rat and human cell lines. Cyclin E functions to regulate the timing of S phase entry and centrosome duplication in cells. Cyclin E overexpression is an indicator of poor prognosis in cancer patients; however the selective advantage gained by deregulating cyclin E has not yet been explained. We propose that in addition to delaying S phase, cyclin E deregulation also causes a delay in mitosis. Therefore, cyclin E may be interfering with mitotic division leading to chromosome instability and eventual tumorigenesis. In this first year of funding, I have accomplished my goals to observe and define the proposed mitotic delay using flow cytometry, immunofluorescence, and live cell microscopy. Cells were found delay in mitosis, specifically in prometaphase. In addition, we are now using biochemical assays to analyze expression of important mitotic regulators and identify a possible substrate of cyclin E/Cdk2 phosphorylation in mitosis.				
14. SUBJECT TERMS No Subject Terms Provided.				15. NUMBER OF PAGES 10
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Deregulation of the cell cycle is a critical step in tumorigenesis. The cell cycle is regulated through the periodic expression of cyclins. Cyclin E expression is normally limited to the G1/S boundary where it functions to initiate DNA replication and centrosome duplication. Deregulated cyclin E in cell culture causes a premature but elongated S phase (Resnitzky *et al.* 1994). In cyclin E transgenic mice, tumor incidence is increased (Bortner and Rosenberg 1997), particularly with expression of a hyperstable cyclin E mutant (Smith *et al.* under revision). The link between cyclin E and tumorigenesis can be explained by the presence of chromosome instability following constitutive cyclin E expression (Spruck *et al.* 1999). The objective of this study is to identify the mechanism by which deregulated cyclin E induces chromosome instability. We hypothesize that one pathway might be through a delay in mitosis and failure to properly segregate chromosomes. This report describes the observations of this mitotic delay in cyclin E expressing cells and begins to explore the mechanism by which this delay occurs.

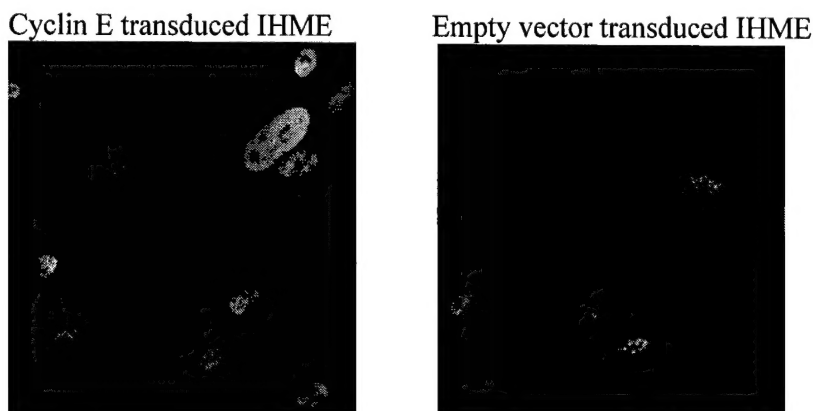
Body

Task 1 - completed: Characterization of cells transduced with cyclin E retrovirus and adenovirus (1-9 months)

a. Cyclin E retrovirus production and infection of IME cells

Cells with ectopically expressed cyclin E were produced to better understand the effects of deregulated cyclin E on the cell cycle. Because standard transfection procedures were ineffective at delivering cyclin E to these non-transformed cells, retroviral and adenoviral constructs were engineered. A cyclin E mutant (T380A) under the retroviral LTR promoter was inserted into the pBabe retroviral construct. This construct was then used to produce retrovirus constitutively expressing hyperstable cyclin E protein. IHME (Immortalized human mammary epithelial) cells were transduced with the retrovirus and selected with hygromycin. Expression of cyclin E was analyzed with immunofluorescence, confirming that all cells expressed cyclin E (many with highly elevated levels). Despite the use of the cyclin E phosphorylation mutant (T380A), protein levels seem to decrease during mitosis. The persistence of cyclin E degradation can be explained by the presence of an alternative phosphorylation site (T62) on cyclin E that allows for some degradation of cyclin E in mitosis. The double mutant (T380, T62) cyclin E has not yet been constructed; however the single mutant cyclin E does appear to affect cell cycle profiles (as seen in Figure 3). Figure 1 shows the expression of cyclin E in IHME cells compared to cells transduced with an empty vector retrovirus.

Figure 1 – Immunofluorescence experiment showing IHME cells following transduction and selection of respective retrovirus constructs. Cells were fixed with methanol and stained with cyclin E (green) and DNA (blue).



To be sure that the ectopically expressed cyclin E was able to bind and activate Cdk2, a kinase assay was performed on histone H1 substrate. Due to the constitutive high levels of Cdk2 found normally in cells, Cdk2 was not a limiting factor.

Figure 2 – Histone H1 kinase assay. IHME and U2OS cells immunoprecipitated with Cdk2. rcyE (retrovirus-transduced cyclin E cells), rcont (retrovirus-transduced empty vector cells). Kinase activity of Cdk2 is greatly increased in rcyE cells.

IHME rcyE IHME rcont U2OS



b. Cyclin E adenovirus production and cyclin E expression

Retroviral delivery of cyclin E and subsequent selection ensures that the total population of cells is expressing the protein; however, this system is best to observe long-term effects of ectopic expression. However, to observe transient and direct effects of cyclin E overexpression an adenovirus is most effective. A wildtype copy of cyclin E was inserted into an adenoviral vector for infection of IHME, U2OS, and KB cells (a cancer cell line derived from HeLa cells). Cyclin E expression was confirmed by Western blot and immunofluorescence.

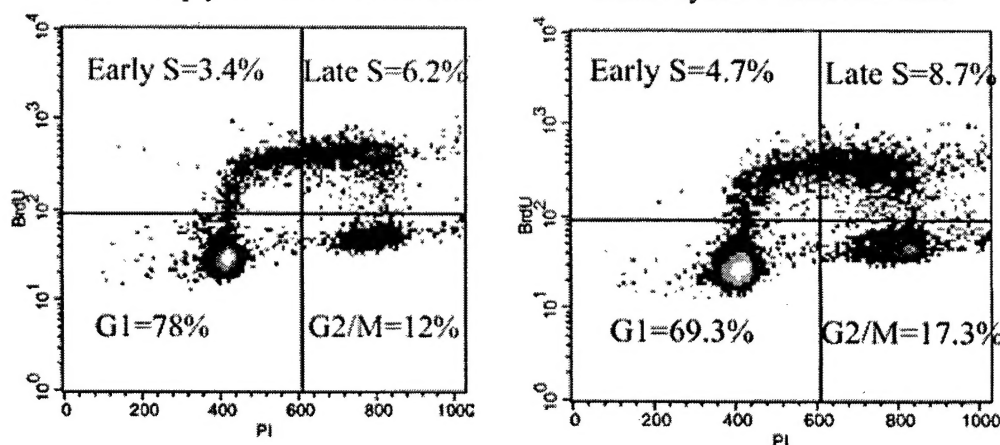
c. Cell cycle analysis

Cells were collected and analyzed by flow cytometry to visualize cell cycle profiles. Using propidium iodide (PI) for cell cycle analysis, a significant increase (10%) in G2/M phases (4N peak) was observed in cells expressing cyclin E compared to cells transduced with empty vector retrovirus. (Data not shown). To more accurately analyze the phases of the cell cycle by flow cytometry, BrdU (allows for the identification of S phase cells) was incorporated into asynchronously cycling cells for 20 minutes before collection. Cells were analyzed by flow cytometry for PI (DNA content) and BrdU (actively replicating cells). These experiments demonstrated a delay in S phase and G2/M phases (17% compared to 12%) (Figure 3b).

Figure 3–Flow cytometry (BrdU and PI staining) showing accumulation of cells in S and G2/M.

IHME empty vector retroviral cells

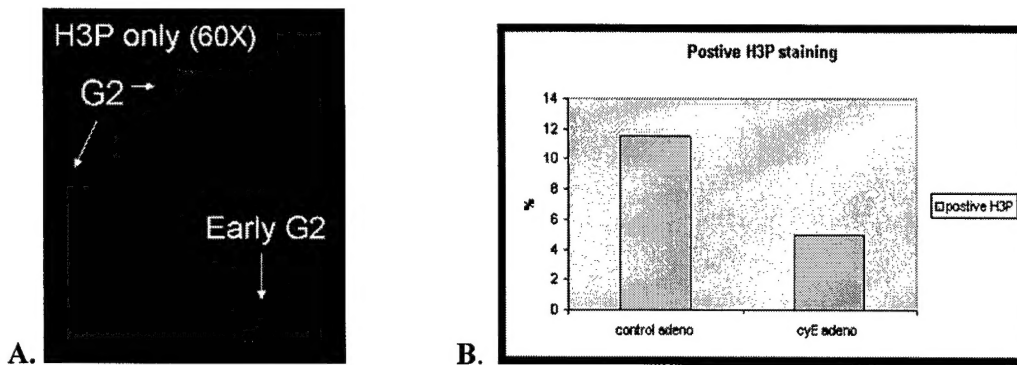
IHME cyclin E retroviral cells



e. *Laser scanning cytometry experiments with H3P and DNA staining*

This experiment was proposed to distinguish G2 from mitotic cells. Cells were plated on coverslips and stained with PI for DNA and histone 3-phosphorylated (H3P) to identify G2 cells. Laser scanning cytometry would provide data similar to flow cytometry but allows the user to visualize individual cells of interest that were selected on a dot graph. Unfortunately, the laser scanning cytometer gave unreliable data in preliminary studies due to cell clumping causing ineffective gating and difficulty in distinguishing G2 staining from mitotic staining. To better analyze G2 cells via H3P staining, immunofluorescence followed by manual counting of H3P-positive cells was used in lieu of laser scanning cytometry. Immunofluorescence is an ideal method of distinguishing G2 cells due to the distinct punctate patterns found in cells throughout G2 (see Figure 4). Asynchronously growing cells were infected with control or cyclin E adenovirus and processed for immunofluorescence. 600 cells were counted and 5% of cells expressing cyclin E were in G2 while 11% of control cells were found to be in G2 (Figure 4). This demonstrates that deregulated cyclin E cells are not delaying in G2. Therefore the increased 4N peak observed by flow cytometry must be due to cells delaying in mitosis.

Figure 4 - A.) Immunofluorescence showing H3P (red) positive cells, counterstained with DAPI for DNA. B.) Graph showing percentage of H3P positive cells, control vs. cyclinE cells.



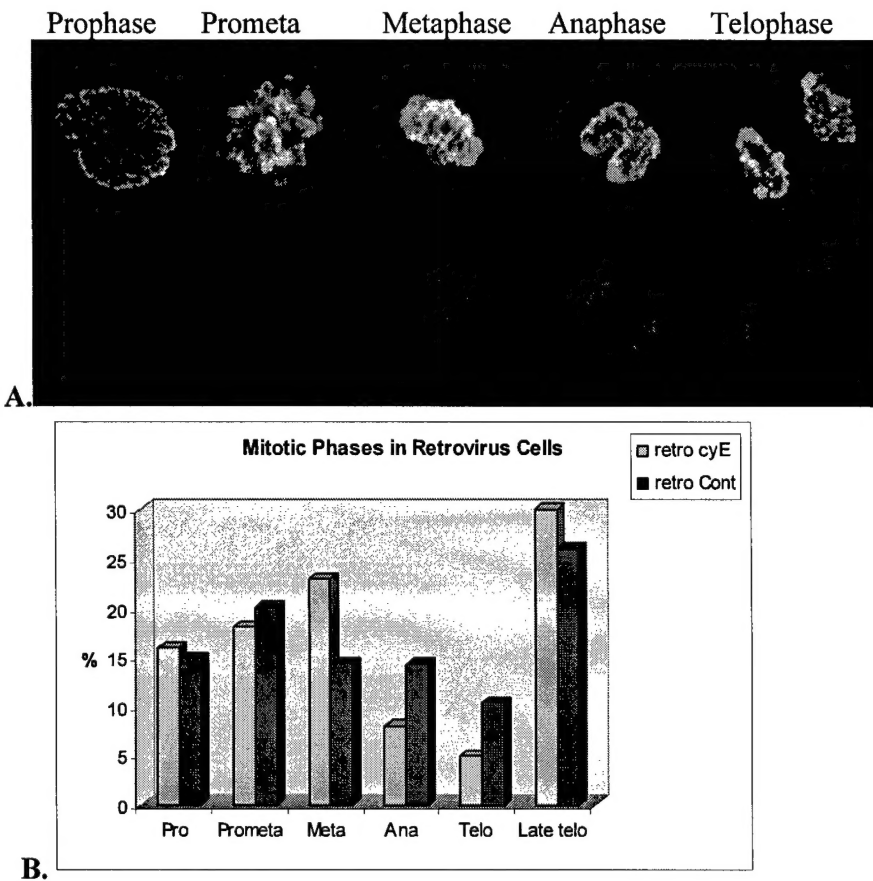
Task2 – preliminary data completed: Observation of aberrant mitosis using live cell microscopy (Months 8-17)

a. *Perform synchrony experiments with thymidine: time release from S phase and appearance of mitotic cells in fixed cells with immunofluorescence*

Analysis of the timing of mitosis in cells synchronized with thymidine and then stained for H3P and Dm1a (alpha microtubules) are on-going. However, experiments were done to analyze the percentage of cells in each of the mitotic phases using immunofluorescence on asynchronous cells. Data from the flow cytometry experiments revealed a slight delay in mitosis. To further explore this delay, experiments were done to identify which mitotic phase was delayed. This could provide insight into the mechanism through which cyclin E is interfering with mitosis. Asynchronous cells were infected with adenovirus, fixed and stained for H3P and Dm1a, and the number of cells in each phase of mitosis was manually counted. The exact mitotic phase was determined by the position of the chromosomes (stained with H3P) and the

orientation of the mitotic spindle (stained with Dm1a). This data was used to supplement the live microscopy data on the timing of mitosis. The primary mitotic delays appear to be in metaphase and prometaphase, corresponding to the activation of the mitotic checkpoint. This data further substantiates our hypothesis that cyclin E might be affecting the spindle checkpoint.

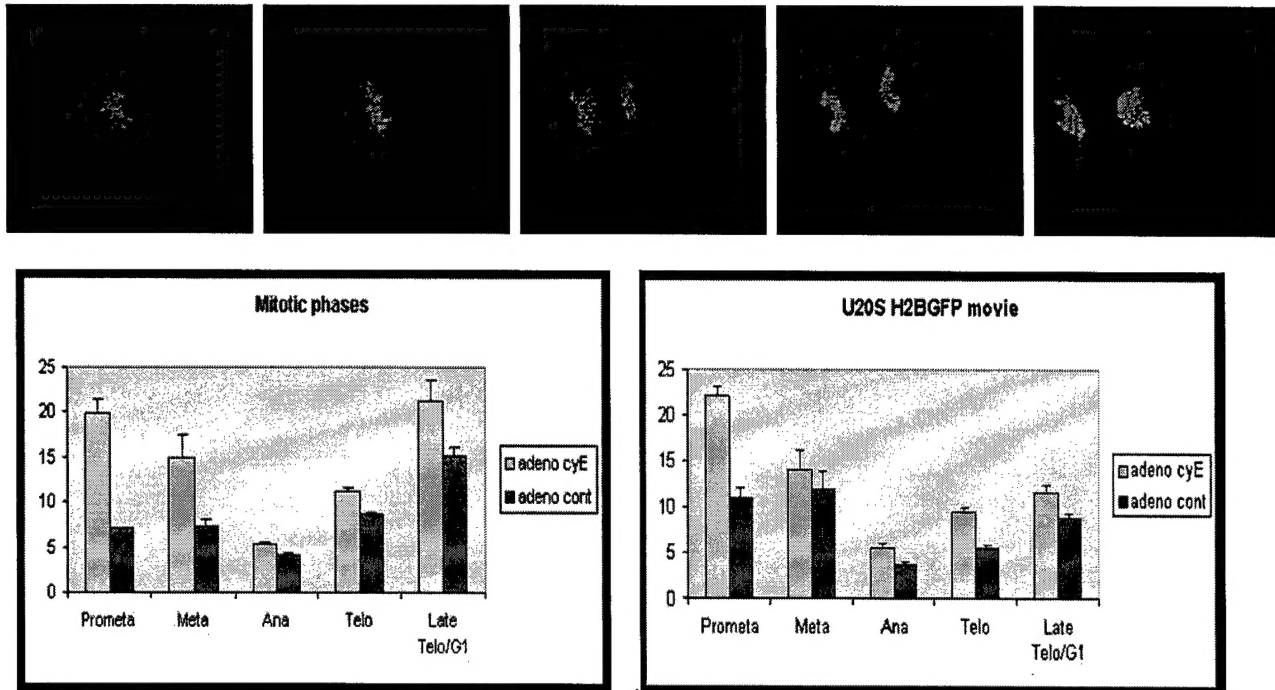
Figure 5 – A.) Immunofluorescence showing each mitotic phase in IHME cells. H3P (green) and Dm1a (red). B.) Graph showing percentages of cells observed in the various phases of mitosis.



b.,c.,d. Film mitosis in live cells expressing H2B-GFP.

After constructing a retrovirus expressing histone 2B-GFP, this retrovirus could be used for expression of H2B-GFP in several cell lines. IHME and U2OS cells were filmed and analyzed to determine the timing of mitosis in cyclin E-expressing cells and empty vector cells. Preliminary data in these cells lines showed that cyclin E tends to delay cells in prometaphase and interferes with progression into anaphase and telophase, as shown in Figure 6.

Figure 6 – Live microscopy experiments using IHME and U2OS cells expressing H2B-GFP for better visualization of the mitotic phases. DIC and fluorescence images were recorded on a deconvolution microscope every minute until the completion of mitosis. Five 3-micron sections were taken. A.) Example of a cell filmed during mitosis, chromosomes are in green (H2B-GFP). B.) Graphs showing the percentage of time (mins) cells spend in each phase of mitosis. Times of each phase were averaged from several movies.



Task 3 - incomplete: Quantification of centrosomes in cells transduced with cyclin E (Months 17-23)

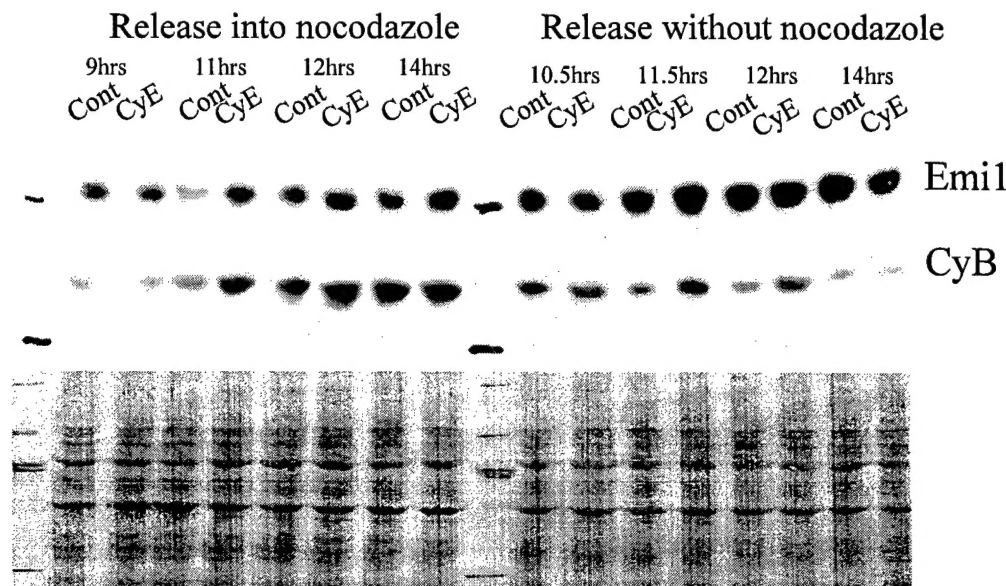
These objectives will be addressed in the second year of this project.

Task 4 – have begun studies: Investigate potential targets of cyclin E/Cdk2 phosphorylation in mitosis (Months 24-36)

I have begun to address this objective but experiments are on going. My preliminary data focuses on the expression of several regulatory proteins throughout mitosis such as cyclin B, cyclin A, and Emi1. Emi1 is a possible cyclin E/Cdk2 substrate that was identified as an F-box protein and is capable of inhibiting the APC (Anaphase promoting complex) in S and G2 (Florence Margottin-Goguet et al. 2003). Upon Emi1 binding to the APC, Cdc20 and Cdh1, which target the APC ubiquitin complex to its substrates, no longer have access to bind and activate the APC. Previous studies have shown that indestructible Emi1 causes delays in prometaphase, consistent with the delays I have observed.

In these experiments, cells were synchronized using a double thymidine block and then released into mitosis. Various time points were collected as cells progress through G2 and mitosis. Expression levels of cyclin B and Emi1 are compared in cyclin E-transduced cells and empty vector-transduced cells. Figure 7 shows accumulation of Emi1 and cyclin B in cyclin E-expressing cells after cell synchrony and release into mitosis.

Figure 7 – Western blot (and amido black for loading comparisons) showing accumulation of Emi1 and cyclin B in mitosis after a double thymidine block and release. Time points were collected for analysis at the indicated times. Cells were released with and without nocodazole. Emi1 and cyclin B are expressed at higher levels in several time points, especially in the presence of nocodazole.



Key Research Accomplishments

- Production of retrovirus and adenovirus constructs for ectopic cyclin E expression in cell culture
- Flow cytometry experiments showing delay in S phase and G2/M phases
- Further investigation of delay using immunofluorescence:
 - Counting of G2 cells
 - Identification of mitotic phase in which cyclinE cells are delayed compared to control cells
- Live cell microscopy to observe mitosis directly and confirm mitotic delays
- Biochemistry experiments to identify possible cyclin E substrates in mitosis and accumulations of key mitotic regulator proteins.

Reportable Outcomes

- Establishment of IHME cell lines with constitutive expression of cyclin E, empty vector control, or H2B-GFP
- Presentation of research results at the Cell Biology of Cancer meeting given by The British Society of Cell Biology and The British Association for Cancer Research, University of Oxford, England – October 2003.
- Presentation of research results at the graduate student retreat for The Scripps Research Institute September 2003.

Conclusions

All objectives for the first year of this study have been accomplished. I have confirmed my hypothesis that deregulated cyclin E can lead to a delay in mitosis with several different techniques such

as flow cytometry, immunofluorescence and live cell microscopy. This delay is slight but consistent and over time the cyclin E expressing cells accumulate a large population of polyploid cells with obvious mitotic defects. However, it is important to study the direct effects of deregulated cyclin E expression using biochemical methods in order to accurately identify the mechanism by which cyclin E interferes with mitosis. These experiments will continue through the next year.

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